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Genetically modified porcine split-thickness skin grafts as an alternative to allograft for provision of temporary wound coverage: preliminary characterization

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ABSTRACT

Temporary coverage of severely burned patients with cadaver allograft skin represents an important component of burn care, but is limited by availability and cost. Porcine skin shares many physical properties with human skin, but is susceptible to hyperacute rejection due to preformed antibodies to α-1,3-galactose (Gal), a carbohydrate on all porcine cells. Our preliminary studies have suggested that skin grafts from α-1,3-galactosyltransferase knock out (GalT-KO) miniature swine might provide temporary wound coverage comparable to allografts, since GalT-KO swine lack this carbohydrate. To further evaluate this possibility, eight non-human primates received primary autologous, allogeneic, GalT-KO, and GalT + xenogeneic skin grafts. Additionally, secondary grafts were placed to assess whether sensitization would affect the rejection time course of identical-type grafts. We demonstrate that both GalT-KO xenografts and allografts provide temporary coverage of partial- and full-thickness wounds for up to 11 days. In contrast, GalT + xenografts displayed hyperacute rejection, with no signs of vascularization and rapid avulsion from wounds. Furthermore, secondary GalT-KO transplants failed to vascularize, demonstrating that primary graft rejection sensitizes the recipient. We conclude that GalT-KO xenografts may provide temporary coverage of wounds for a duration equivalent to allografts, and thus, could serve as a readily available alternative treatment of severe burns.

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1. Introduction

Early excision of burned tissue and replacement with autologous skin grafts is a mainstay of burn treatment, and has been shown to reduce patient mortality by preserving the skin's barrier function, preventing fluid loss and subsequent hypovolemia, electrolyte, temperature and pH imbalances that if untreated contribute to infection, multisystem organ failure, and death. However, the supply of uninjured skin may be limited in severe burns; therefore, alternative means of temporary coverage to preserve barrier function are needed [1–8].

Cultured autologous keratinocytes and various artificial dermal substitutes have been described to this end, however these approaches have significant disadvantages and the outcomes are inferior to allogeneic skin grafts [9–13]. Cultured autologous keratinocytes require weeks to grow before application and yield a thin, delicate graft that is easily injured [10,13]. Artificial dermal substitutes (such as Biobrane™, Transcyte™, and Integra™) are expensive, require vessel ingrowth (a process that can take up to 2–3 weeks) for optimal protection against infection, and still require autologous skin grafts to achieve permanent wound closure [9,14–18]. Surfrasoft™, Mepitel™, and Suprathel™ have mostly been used as biologic dressings to aid in the healing of partial-thickness burn wounds or to aid in healing of split-thickness skin grafts [19–21]. EZ derm, a biosynthetic derived from porcine dermis, has been studied in a few clinical settings as a biosynthetic dressing for partial-thickness burns [22,23] The EZ derm provided wound coverage for about 5–7 days and sloughed as the underlying wound epithelialized [22,23]. Full-thickness wounds still require definitive closure with autologous skin grafts.

The current gold standard for temporary coverage of full-thickness burns is allogeneic cadaver skin. While allogeneic skin grafts predictably reject from the wound bed 7–12 days after placement due to immunologic incompatibility between the burn victim and the cadaver donor, allografts undergo vascularization within 2–3 days in a manner similar to autologous grafts, and are therefore viable and capable of providing a barrier in the early post-burn time frame, when protecting against infection and physiologic insults associated with the loss of skin integrity. Despite their effectiveness, however, allografts also have disadvantages, including cost, limited availability, and the risk of pathogen transmission [24].

Xenogeneic skin grafts provide a potential alternative for temporary wound coverage. Porcine skin has considerable similarity to human skin that makes it an attractive option for temporary wound coverage and maintaining barrier functions in the early post-burn period; including structurally similar rete ridges, papillary dermis, and sparse hair coverage [14,25–28]. In addition, swine share few pathogens in common with humans, thus reducing the risk of disease transmission when compared with cadaveric grafts [29–31]. Furthermore, it would be possible to maintain a herd of swine in a climate-controlled, pathogen-free environment for the purpose of skin graft procurement, an important practical consideration in ensuring consistent availability of high-quality skin suitable for use in medical settings.

Historically, porcine skin grafts have not been a viable option, as they fail to vascularize due to hyperacute rejection, an immediate attack on the endothelium of graft blood vessels mediated by preformed antibodies in humans and Old World primates against the α-1,3-galactose (Gal) moiety present on swine cell membranes [32,33]. Antibody-mediated endothelial injury results in a diffuse thrombotic microangiopathy and subsequent ischemic insult, resulting in quick desiccation and avascular necrosis. Thus, the barrier function of the graft fails after a few days and may even serve as a nidus for bacterial colonization or superinfection.

To avoid the problem of hyperacute rejection, genetically-modified swine have been prepared that do not express the Gal epitope due to selective knockout of the gene encoding α-1,3-galactosyltransferase (GalT-KO) [34]. The availability of these animals now makes it possible to carry out pig-to-primate xenografts without hyperacute rejection mediated by anti-Gal antibodies. Solid organ transplantation from pig-to-primate using GalT-KO swine did not show hyperacute rejection and had prolonged organ survival compared to Gal normal swine [35,36]. Preliminary studies performed in our laboratory have suggested that skin grafts from GalT-KO swine may survive as long as allografts on baboons [25]. Here we have further studied GalT-KO skin grafts to evaluate their performance in comparison with allografts as a potential alternative treatment options for severely burned patients. We demonstrate that skin grafts from GalT-KO miniature swine engraft on primates and provide temporary wound coverage for a period comparable to that offered by allogeneic skin and considerably longer than wild type GalT + porcine grafts.

2. Methods

2.1. Animals

This study was approved by the Massachusetts General Hospital (MGH), Institutional Animal Care and Use Committee (IACUC) and performed in accordance with the guide for the care and use of laboratory animals [37]. Eight baboons (*Papio hamadryas*) were obtained from Mannheimer Foundation, Inc, Homestead, FL. All baboons were aged 2–5 years and weighed 6–10 kg each. The animals underwent routine pathogen screening and quarantine prior to commencement of the studies.

Genetically engineered GalT-KO miniature swine were produced in our own swine facility [34]. The GalT-KO swine herd is monitored using a computerized system to ensure availability and quality control, and housed in a purpose-built facility, which is fully integrated as part of the animal facilities in the laboratory with input and veterinary oversight from the Center for Comparative Medicine of the MGH.

2.2. Skin graft harvest

Swine donors were anesthetized with 2 mg/kg Telazol intramuscular (IM) injection, intubated, and anesthesia maintained using 2% isoflurane and oxygen. The skin surface was disinfected before surgery with 2% (w/v) chlorhexidine

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acetate (Nolvasan[R] Surgical Scrub, Fort Dodge Animal Health, Fort Dodge, IA), 70% isopropyl rubbing alcohol (Nolvasan[R] Surgical Scrub, Owens & Minor, Mechanicsville, VA), and povidone-iodine, 10% (Betadine Solution, Purdue Products, L.P., Stamford, CT) scrubs. The animal was then draped leaving the dorsum exposed. Split-thickness skin grafts were harvested using an air-driven Zimmer dermatome (Medfix Solution, Inc., Tucson, AZ, USA) with the depth set to 0.022 inches. Following harvest, the skin specimens were stored at 4°C until preparation of the recipient site. Grafts that were not used immediately were cryopreserved and stored at −80°C until required (between 1 week and 6 months). In our experience, we have seen that frozen and fresh skin grafts engraft comparably, so for convenience, mostly frozen grafts were used in this study. Hemostasis was achieved with gauze soaked in saline and epinephrine and the wound was dressed with Telfa[TM] non-adhesive dressing (Covidien, Dublin, Ireland) and Tegaderm[TM] (3 M, St. Paul, MN).

Baboon donors were treated with 0.1 mg/kg atropine IM and 20 mg/kg ketamine IM and transferred to the operating room. The dorsum was shaved using clippers prior to entering the operating room. Endotracheal intubation was performed and anesthesia maintained with 2% isoflurane and oxygen as described above. The dorsum of the donor baboon was disinfected, draped, and skin grafts were harvested and stored as described above.

2.3. Wound preparation and skin graft placement

Baboon recipients were pre-medicated, shaved, anesthetized, and prepped as described above. Partial-thickness defects measuring 4 × 5 cm were prepared by passing an air-driven Zimmer dermatome over the skin, set to 0.033 inches. Full-thickness defects measuring 4 × 5 cm were prepared by sharp excision of skin, subcutaneous tissue, and fascia down to the muscle. Split-thickness skin grafts were fenestrated then sutured in place over both partial-thickness and full-thickness wounds using an interrupted 2–0 silk sutures. Each animal received four grafts: autologous, allogeneic, GalT-KO, and GalT + . Bacitracin ointment was applied and the grafts were dressed with Telfa[TM] non-adhesive dressing and Tegaderms[TM]. Sterile dry gauze was then applied over the grafts and secured in place with Vetrap[TM] (3 M, St. Paul, MN) bandaging tape to create pressure dressings. Recipients were then dressed with cotton jackets to reduce interference with the grafts.

2.4. Clinical and histological assessment

Recipients underwent evaluation first on POD 4, then every 2–3 days thereafter. Skin grafts were assessed for viability and integrity, and determined to be rejected when less than 10% of viable graft tissue covered the wound [38]. Median graft survivals were compared using a log-rank test in GraphPad Prism, version 5.04 for Windows, GraphPad Software (San Diego, CA). Six mm punch biopsies were taken from viable areas of the grafts at 4-days interval post-operatively, and hematoxylin and eosin (H&E) stained slides were evaluated for features of rejection by a pathologist blinded to specimen identity.

2.5. Immunosuppression

Cyclosporine A was administered intramuscularly in selected recipients at a dose of 13–15 mg/kg once daily. Blood levels were monitored daily 12 h after the previous dose and the dose adjusted to achieve a target trough between 400 and 600 ng/mL.

3. Results

3.1. Xenogeneic skin grafts from GalT-KO miniature swine exhibit comparable survival to allografts

Four baboons received a combination of autologous, allogeneic, GalT-KO, and GalT + xenogeneic split-thickness skin grafts on separate partial-thickness dorsal wounds (Fig. 1A). All wounds and skin grafts were standardized to 4 × 5 cm, confirming the results of our previous preliminary study [25]. Autologous grafts survived indefinitely, while allogeneic and GalT-KO grafts survived a median of 11 days (Fig. 1B). Because the skin grafts were only observed on alternate days post-operatively, our failure dates have an error of +/−1 day. It is possible that the grafts failed on different days within a 2-day window, but were only declared failed during the designated observation period. GalT + xenogeneic grafts were hyperacutely rejected in 4 days (Fig. 1B). All grafts were warm and adherent by POD 4 with the exception of the GalT + grafts, which appeared to be hyperacutely rejected (Fig. 1C–F). Autologous skin grafts healed and exhibited healthy epidermis and dermis on histological examination (Fig. 1G). At POD 4, all allografts and GalT-KO xenografts were intact, viable grafts with mild epidermal spongiosis (Fig. 1H,I). GalT-KO xenografts had evidence of revascularization, with intravascular red blood cells and focal intravascular fibrin (Fig. 1I). In contrast, the GalT + xenografts had the clinical appearance of ''white grafts'' at the first graft check on POD 4, without clinical or histologic evidence of revascularization, which is consistent with hyperacute rejection. GalT + xenografts showed endothelial injury, epidermal vacuolization and apoptosis, and ischemic injury on histopathology, and were lost shortly thereafter (Fig. 1J).

To better approximate severe burns or blast injuries requiring grafting, four baboons had full-thickness wound beds prepared that were grafted with autologous, allogeneic, GalT-KO, or GalT + skin grafts as described above for partial-thickness wounds (Fig. 2A). Similar to partial-thickness wounds, autologous grafts survived indefinitely on full-thickness wounds (Fig. 2B). Autologous grafts, as expected, demonstrated evidence of complete clinical and histological healing (Fig. 2C). Allografts and GalT-KO xenografts behaved similarly to those placed on partial-thickness wounds, with early engraftment and histological evidence of recirculation, but progression to rejection by POD 11 (Fig. 2B,C). GalT + skin grafts were again found rejected at first check on POD 4, with the clinical appearance of ''white grafts'' that had not vascularized (Fig. 2B,C).

3.2. Second set xenogeneic skin grafts from GalT-KO miniature swine undergo accelerated rejection

Repeat grafting with multiple sequential graft or skin substitute applications while donor sites to heal sufficiently for

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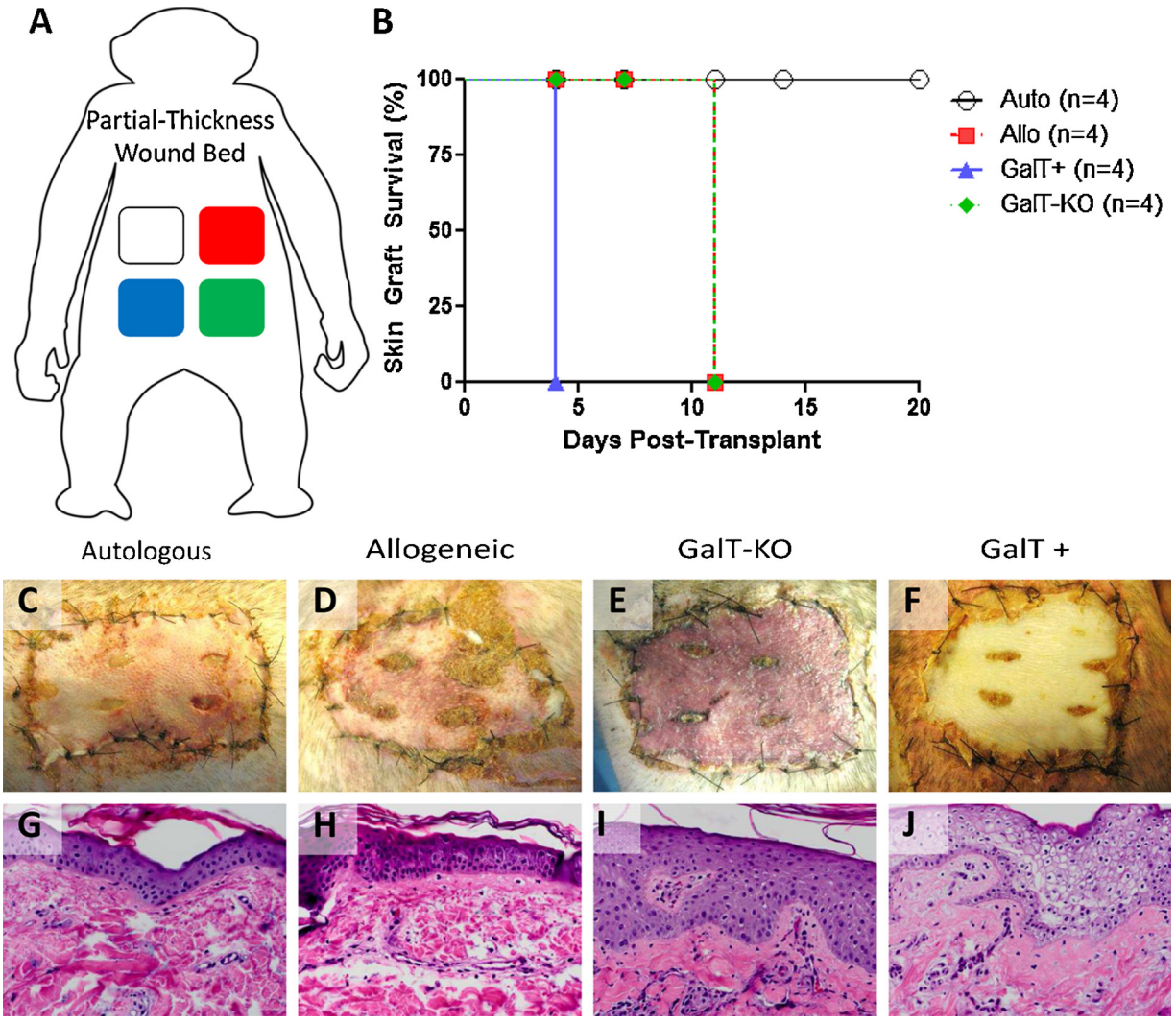

Fig. 1 – (A) Experimental design demonstrating the types of split-thickness skin grafts (autologous-black outline, allogeneic-red, GalT-KO xenogeneic-blue and GalT + xenogeneic-green) placed on partial-thickness wound. (B) Survival of autologous, allogeneic, GalT-KO xenogeneic and GalT + xenogeneic split-thickness skin grafts on partial-thickness wound beds. Gross clinical photos taken on POD 4 of autologous (C), allogeneic (D), GalT-KO xenogeneic (E) and GalT + xenogeneic skin grafts (F). The respective histology images are shown from biopsies taken on POD 4 of autologous (G), allogeneic (H), GalT-KO xenogeneic (I), and GalT + xenogeneic (J) skin grafts.

re-harvest may be necessary to achieve ongoing wound coverage during the treatment of a severely burned patient who has limited uninjured skin for autologous grafting. Repeated applications of immunologically identical material could result in recipient sensitization and cause accelerated cellular or antibody mediated rejection of xenografts. To evaluate this possibility, a second set of allogeneic and GalT-KO xenogeneic skin graft was placed on four previously grafted recipients (Fig. 3A). Following complete rejection of all first set skin grafts, second set grafts were placed on freshly debrided full-thickness wounds. The secondary autologous grafts survived indefinitely, but the allogeneic and GalT-KO secondary grafts rejected earlier than primary grafts, at a median of 6.5 and 4 days, respectively (Fig. 3B). Second set GalT-KO grafts presented as ''white grafts,'' with no evidence of vascularization, and were found avulsed when first examined at POD 4 (Fig. 3C). Interestingly, while second set allogeneic skin grafts also rejected in an accelerated fashion in comparison to primary grafts (complete rejection by POD 7), these grafts did not have the appearance of a white graft (Fig. 3C), and were rejected in accelerated-acute rather than hyperacute fashion. When we assessed the presence of anti-GalT-KO antibodies following rejection of the first set of grafts, we found evidence of both GalT + and GalT-KO antibodies, which signifies that the primary skin grafts sensitized the recipients (Fig. 4).

3.3. *Systemic immunosuppression failed to prolong skin graft survival*

Recognizing that survival of allogeneic skin grafts on burn recipients may be markedly prolonged, presumably due to a

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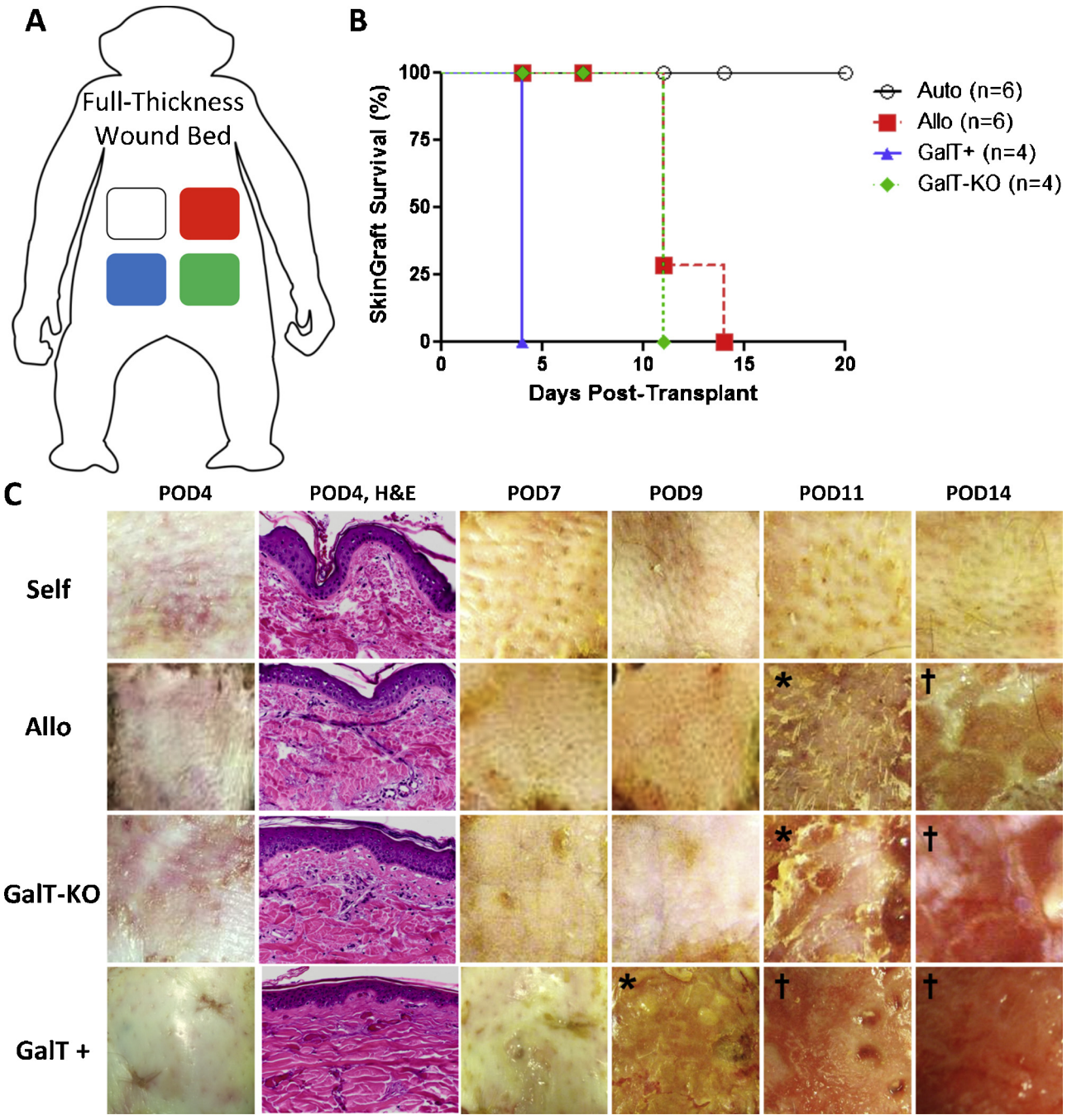

Fig. 2 – (A) Experimental design demonstrating the types of split-thickness skin grafts (autologous-black outline, allogeneic-red, GalT-KO xenogeneic-blue and GalT + xenogeneic-green) placed on full-thickness wound beds. (B) Survival of autologous, allogeneic, GalT-KO xenogeneic and GalT + xenogeneic split-thickness skin grafts on full-thickness wound beds. (C) Gross clinical results at various PODs illustrating the rejection time course of allogeneic, GalT-KO xenogeneic, and GalT + xenogeneic skin grafts on full-thickness wounds. A time course of autologous skin grafts is included for comparison. Representative histology images are included from POD 4 biopsies. (* represents a rejected graft; † represents an avulsed graft).

degree of systemic immune-compromise associated with the injury [39]. To prolong the duration of temporary wound coverage, we investigated the ability of monotherapy immunosuppression with Cyclosporine A to prolong survival of GalT-KO skin grafts. Four baboons received a combination of autologous, allogeneic, GalT-KO, and GalT + split-thickness skin grafts and were treated with systemic Cyclosporine A with trough levels of 400–600 ng/mL. While therapeutic levels of Cyclosporine A were attained, survival of the skin grafts was not prolonged. GalT-KO xenografts had equivalent survival (mean 11 days) on both untreated and treated baboons ($p = 1$), allogeneic skin grafts survived an average of 11 days on

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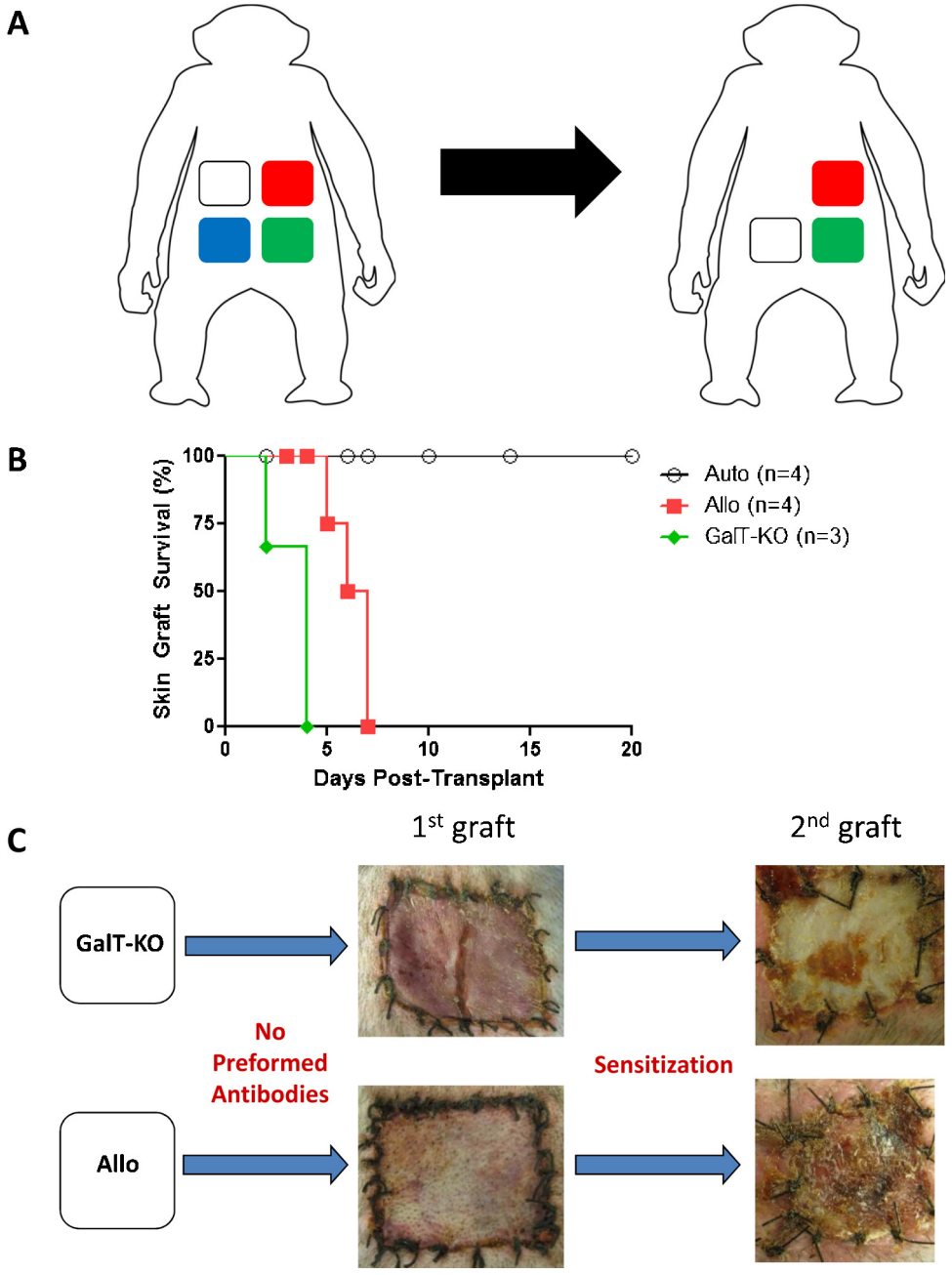

Fig. 3 – (A) Experimental design demonstrating that four monkeys received four split-thickness skin grafts (autologous-black outline, allogeneic-red, GalT-KO xenogeneic-blue and GalT + xenogeneic-green) placed on full-thickness wound beds. Once the primary grafts rejected, all wounds except the autologous grafted wounds were debrided to healthy muscle then received repeat identical split-thickness skin grafts (autologous-black outline, allogeneic-red, and GalT-KO xenogeneic-blue). (B) Survival of the second set identical allogeneic and GalT-KO xenogeneic grafts is significantly shorter than the first round of grafting due to immunological sensitization. Initial allogeneic and xenogeneic GalT-KO split thickness skin grafts remained viable until POD 11; subsequent identical GalT-KO grafts reject by POD 4. (C) Gross clinical photos illustrate the accelerated rejection due to sensitization from subsequent allogeneic and GalT-KO xenogeneic skin grafts.

baboons that were treated with systemic Cyclosporine A and 11.5 days on untreated baboons ($p = 0.54$) (Table 1). Calcineurin inhibitor monotherapy is unlikely to control hyperacute rejection, which is mediated by preformed antibodies, and as expected, survival of GalT + xenografts was not extended by immunosuppression (Table 1).

4. Discussion

Why primates developed anti-Gal antibodies remains unknown; however, current hypotheses suggest that these antibodies were produced by exposure to sugar moieties on

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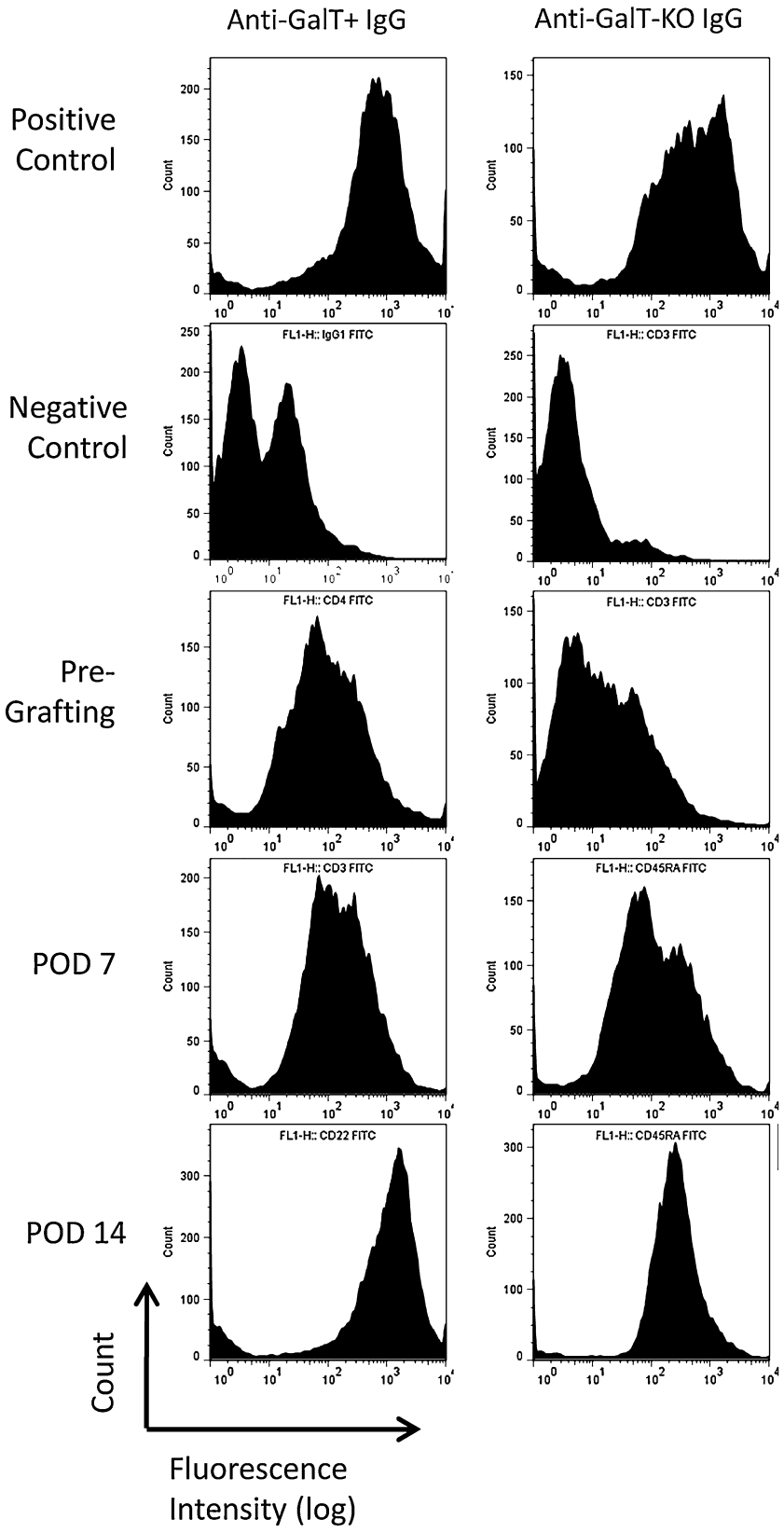

Fig. 4 – The fluorescence-activated cell sorting (FACS) plot on the left demonstrates pre-formed antibodies to GalT + with an increase in antibody production following GalT + skin graft placement (right shift of the peak). The FACS plot on the right demonstrates no pre-formed antibodies to GalT-KO, but formation of antibodies after split thickness skin graft placement (right shift of the peak from the negative control range).

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Table 1 – Survival of allografts on baboons treated with systemic immunosuppression (IS) was not prolonged compared to grafts on untreated baboons. GalT-KO and GalT + xenogeneic grafts also survive the same duration whether the baboon was treated with systemic IS or not.

Graft type	Mean survival without IS (in days)	Mean survival with IS (in days)	*p*-value
Allografts	11.5	11	0.54
GalT-KO Xenografts	11	11	1.0
GalT + Xenografts	4	4	1.0

intestinal bacteria, as anti-Gal antibodies cross-react with many such moieties. Regardless of the nature of their origin, anti-Gal antibodies in primates, unfortunately (for xenotransplantation scientists) mediate hyperacute rejection of swine organs transplanted to primates [40,41]. Although humans and other Old World primates have antibodies against swine antigens other than Gal (i.e. ''non-Gal'' swine antigens), organ transplantation studies in our center have confirmed that these non-Gal antibodies are no more prevalent or toxic than allogeneic antibodies, such as those encountered in transplantation between members of the same species [42].

These experiments confirm previous demonstrations that primate recipients of skin grafts from wild-type pigs expressing the Gal antigen (GalT +) reject these grafts hyperacutely due to the presence of preformed anti-Gal antibodies in primates [25,32,33]. We have confirmed our preliminary results showing that skin grafts from GalT-KO swine have commensurate survival times with the typical survival of MHC-mismatched allograft skin [25]. The previous study utilized only two animals that had previously undergone prior experiments, which included treatments causing temporary immunosuppression, and they were treated with systemic Cyclosporine A while the skin grafts were in place [25]. Also, the skin grafts were placed on partial-thickness wound beds only [25]. In the current study, we observed comparable graft survival times for GalT-KO and allogeneic grafts on both partial- and full-thickness wounds. These findings suggest that skin grafts from GalT-KO miniature swine could serve as an alternative to cadaveric allogeneic skin.

It is not uncommon for patients suffering large burns to require multiple skin grafts from the available donor sites, which require a sufficient interval for healing to occur between each successive graft harvest. In the allogeneic setting, second graft survival could be potentially prolonged by using a different donor with a separate MHC type, but sensitization to pig-specific antigens may preclude the use of subsequent porcine xenografts. We observed that placement of skin grafts (either allogeneic or GalT-KO xenogeneic grafts) sensitizes the recipient and second grafts of the same type undergo hyperacute, or accelerated-acute rejection, as seen in allogeneic skin grafts in many other models and in clinical practice [43–47]. Thus, while primary xenografts function equivalent to primary allografts, these data suggest that they could only be used once in a given patient.

To investigate whether or not primary xenografts could provide extended coverage, we treated recipients with systemic immunosuppression. Despite achieving trough concentrations of Cyclosporine A in blood within a therapeutic range previously shown to induce long-term survival of solid organ allografts across a Class I allogeneic barrier, this was not sufficient to prolong survival of any of the skin grafts placed [38]. Allotransplanted skin is known to be one of the most highly antigenic tissues [48], this result may reflect the potent immunogenicity of skin, and a higher level of immunosuppression may be required to prevent rejection of split-thickness skin grafts. However, treating patients already at risk of infection as a result of burn injury with substantial doses of immunosuppression would not be a practical or appropriate approach to extending the survival of temporary skin grafts. An alternative strategy would be placement of GalT-KO and allogeneic grafts in series, and experiments investigating this are ongoing [49].

GalT-KO porcine skin grafts offer several practical advantages as temporary grafts in the management of burn injury: porcine skin is comparable to human skin histologically and functionally. The data reported herein suggest that skin grafts from GalT-KO miniature swine could provide effective, temporary closure of full-thickness wounds analogous to those experienced by severe burn and blast victims. In contrast to allogeneic cadaver skin, which can often be in limited supply, a commercial herd of GalT-KO miniature swine could readily be maintained ensuring reliable availability and reduced costs. We have demonstrated that GalT-KO skin performs equivalent to allogeneic skin in the experiments above, and could be considered a viable alternative to cadaveric skin grafts in severely burned patients.

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